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<p>(54) Title: SERUM-FREE MEDIUM FOR THE PROLIFERATION OF LYMPHOKINE-ACTIVATED KILLER CELLS</p> <p>(57) Abstract</p> <p>Production of lymphokine activated killer cells. The invention discloses a serum-free culture medium capable of producing such cells with high efficiency.</p>		

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SERUM-FREE MEDIUM FOR THE PROLIFERATION OF
LYMPHOKINE-ACTIVATED KILLER CELLS

FIELD OF THE INVENTION

The invention pertains to compositions suitable for the in vitro culturing of lymphokine-activated killer cells. Specifically, the invention discloses a serum-free medium capable of permitting the proliferation of such cells.

BACKGROUND ART

One of the most promising approaches in the treatment of cancer involves the concept of passive immunotherapy. This approach involves the transfer to a cancer patient of previously sensitized immunologic reagents, such as cells or antibodies, that have the ability to provoke an anti-tumor response (Rosenberg, S.A., Canc. Treat. Rep., 68:233-255 (1984)). Among the many possible immunologic reagents which may be administered, are lymphokines, such as interleukin-2 (IL-2) or interferon. Unfortunately, cancer patients are typically immunosuppressed, and consequently, the success of immunotherapy has been more limited than had been hoped (Rosenberg (1984)). Recently, Grimm and co-workers identified a novel approach to immunotherapy which has significant potential for overcoming

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this drawback (Grimm, E.A., et al., J. Exper. Med., 155:1823-1841 (1982); Grimm, E.A., et al., J. Exper. Med., 157:884-897 (1983); Grimm, E.A., et al., J. Exper. Med., 158:1356-1361 (1983)). These researchers discovered that if peripheral blood lymphocyte (PBL) cells were removed from a patient, and incubated in vitro in the presence of interleukin-2, the cells would acquire the ability to preferentially recognize and destroy tumor cells. This discovery is especially significant in view of the immunosuppressed state of typical cancer patients, in that it enables the physician to withdraw a small number of lymphocytes and to culture these cells in vitro to produce a large number of tumor-specific immunocompetent cells. Since these cells were activated by the presence of a lymphokine to kill tumor cells, they have been referred to as lymphokine activated killer cells (LAK) cells.

Although initial experiments were conducted using natural lymphokines, it has subsequently been discovered that lymphokines produced through recombinant DNA technology are also capable of mediating this activation (Rayner, A.A., et al., Cancer, 55:1327-1333 (1985), Itoh, K., et al., J. Immunol., 134:3124-3129 (1985), Mule, J.J., et al., J. Immunol., 135:646-652 (1985)).

Since PBL cells are naturally present in human blood and lymphatic tissue, it had initially been believed that the administration of a lymphokine to a cancer patient would result in the patient's generation of LAK cells. The generally immune suppressed nature of cancer patients, and the presence of specific factors which suppress LAK cell induction have,

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thus far, blocked this potential therapeutic regimen. Hence, in order to produce LAK cells, it is necessary to cultivate PBL cells in vitro, in the presence of a lymphokine, and then to administer the in vitro derived LAK cells to a cancer patient.

The in vitro generation of LAK cells was originally attempted in culture medium which contained human serum (Grimm, E.A., et al., J. Exper. Med., 157:884-887 (1983)). The extreme cost of such sera, its limited availability and the possibility that it might be contaminated by microorganisms, especially viruses prompted investigations aimed at identifying alternative culture media which would be capable of proliferating the PBL cells and the generation of LAK cells.

Mazumder, A., et al., (J. Exper. Med., 159:495-507 (1984)) and Rosentein, M. et al., (J. Natl. Canc. Inst., 72:1161-1165 (1984)) demonstrated that culture media which contained fetal calf sera was capable of stimulating the production of LAK cells. Fetal calf sera was, however, found to be significantly less effective than human sera in inducing LAK cell formation (Imir, P. et al., Clin. Immunol. Immunopath., 36:289-296 (1985)). These workers found that the number of LAK cells which could be generated in human sera was more than eight times higher than that which an equivalent PBL cell inoculum could produce when cultured in medium containing bovine sera. These results led Imir, et al. to conclude that fetal calf sera appeared to inhibit the generation of LAK cells, and thus that human sera was a preferred culture supplement. Moreover, the use of fetal calf sera appear-

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ed to have a direct toxic effect on either the LAK cell precursors or the LAK cells themselves (Imir, et al., (1985)).

In response to the above-described limitations, Froelich, C.J., et al. (J. Immunol. Meth., 86:205-211 (1986)) identified a serum-free medium capable of both supporting the proliferation of PBL and the generation of LAK cells. The medium identified by these researchers was Dulbecco's Modified Eagle's Medium which was supplemented with bovine serum albumin and the three fatty acids: linoleic acid, oleic acid and palmitic acid. The medium also contained insulin, transferrin, and ethanolamine. This medium was capable of permitting the proliferation of LAK cells, albeit at an efficiency significantly lower than that of human sera-containing media. (Froelich, C.J., et al., (1986)) found that the efficiency of LAK cell generation by this serum-free medium could be enhanced through the addition of sodium pyruvate and indomethacin, an inhibitor of prostaglandin biosynthesis. Even with these supplements, however, the serum-free medium described by Froelich, et al. was found to be significantly less effective than media containing human sera in permitting the proliferation of LAK cells (Froelich, et al. (1986)). In addition, the inclusion of bovine serum albumin to the serum-free medium raised questions as to whether an immune response would be mounted in vitro against the foreign bovine albumin and as a result, indirectly activate LAK cell expansion and activation. Moreover, the possibility that a patient might mount an immune response, in vivo, due to the presence of the foreign bovine albumin raised questions as to the suitability of using bovine

albumin to culture PBL cells which would subsequently be returned to a patient.

The present invention is based upon the discovery that the activation of kinase C, a natural and endogenous enzyme present in PBL cells, permits PBL cells to develop into proliferating LAK cells.

Kinase C is an enzyme which has been found to be the physiologic receptor for phorbol esters, a unique class of tumor promoters (Farrar, W.L., et al., Lymphokine Res., 4:87-93 (1985)). Interleukin-2 has been found to be capable of activating the kinase C enzyme. Activated kinase C, has been found to be capable of phosphorylating the interleukin-2 receptor molecule of intact lymphocytes, suggesting that it may have a role in an animal's immune response (Farrar, et al. (1985) Taguchi, M., et al., Biochem. Biophys. Res. Commun., 135:239-247 (1986)).

Once activated by the lymphokine, LAK cells are capable of proliferating and producing gamma interferon. It is believed that the gamma interferon so produced, interacts in concert with the exogenously added interleukin-2 to further stimulate the production of gamma interferon. The presence of gamma interferon is further believed to be involved in the expression of cellular receptor sites for the interleukin-2 molecule (Shiiba, K., Cancer, Immunol. Immunother., 21:119-128 (1986)). The gamma interferon produced by LAK cells is believed to be responsible for the cytotoxic and therapeutic properties of the cells (Rosenstein, M., et al., Cancer Res. 44:1946-1953 (1984); Rayner, A.A., et al., J. Natl. Canc. Inst. 75:67-74 (1985)).

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Thus, in summary, the ability to treat cancer patients with the LAK cell therapy discovered by Grimm, et al., (1983) requires the use of a cell culture medium which is both economical and effective in permitting the generation of such cells. The use of human sera, though effective in generating LAK cells, is prohibitively expensive, a potential carrier of viruses and of limited supply. Efforts to employ alternative non-human sera or serum-free media have not proven to be wholly satisfactory. Thus, a continued need exists for an alternative, economical and effective culture for generating LAK cells.

SUMMARY OF THE INVENTION

The present invention relates to methods and compositions of matter which permit the generation of LAK cells in a defined, serum-free culture medium. The cell culture medium permits the proliferation of LAK cells at an efficiency which is substantially equivalent to that obtainable through the use of a culture medium which contain human serum. The cell culture medium of the present invention is, however, superior to such serum-containing culture media in that the use of a defined, serum-free medium can facilitate the elucidation of the mechanism which regulates the activation of PBL cells into LAK cells.

In detail, the invention comprises:

A method for achieving the proliferative growth of a peripheral blood lymphocyte cell or a lymphokine activated killer cell, which method comprises:

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- a) contacting the cell with an amount of a culture medium sufficient to permit the in vitro cultivation of the cell, the medium being substantially free of serum and containing a non-proteinaceous compound capable of activating the enzyme, kinase C; the compound being present in the medium at a concentration sufficient for the activation, and
- b) maintaining the cell in the culture medium under conditions sufficient to permit the proliferative growth.

The invention is further directed toward a method for producing a lymphokine activated killer cell which comprises:

- a) contacting a peripheral blood lymphocyte cell with an amount of a culture medium sufficient to permit the in vitro cultivation of the cell, the medium being substantially free of serum and containing (i) a lymphokine and (ii) a non-proteinaceous compound capable of activating the enzyme, kinase C; the compound being present in the medium at a concentration sufficient for the activation, and
- b) maintaining the cell in the culture medium under conditions sufficient to permit the production of the lymphokine activated killer cell.

The invention further pertains to a method for suppressing the growth or development of a cancer cell which comprises:

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- a) contacting a peripheral blood lymphocyte cell with an amount of a culture medium sufficient to permit the in vitro cultivation of the cell, the medium being substantially free of serum and containing (i) a lymphokine and (ii) a non-proteinaceous compound capable of activating the enzyme kinase C; the compound being present at a concentration sufficient for the activation,
- b) maintaining the cell in the culture medium under conditions sufficient to permit the development of a lymphokine activated killer cell, and
- c) providing the lymphokine activated killer cell to the cancer cell in a manner sufficient to suppress the growth or development of the cancer cell.

The invention is further directed toward a composition of matter which comprises: (a) an in vitro cultivated peripheral blood lymphocyte cell or lymphokine activated killer cell, and (b) an exogenously added non-proteinaceous compound capable of activating the enzyme kinase C; wherein the composition of matter is substantially free of serum.

Additionally, the invention pertains to a culture medium which contains an exogenously added non-proteinaceous compound capable of activating the enzyme kinase C; wherein the culture medium is substantially free of serum.

- DESCRIPTION OF THE PREFERRED EMBODIMENTS

I. The In Vitro Cultivation of PBL Cells

A cell is said to be "maintained" in a tissue culture medium when it is cultivated under conditions sufficient to either maintain cellular viability or promote proliferative growth. As used herein, the term "cellular viability" refers to the ability of a cell to metabolize nutrients, and direct biosynthetic processes. The term "proliferative growth" refers to the ability of a cell to produce progeny cells. The maintenance of cellular viability does not require the proliferative growth of the cell.

In general, the in vitro cultivation of the cells of the invention requires a consideration of four basic elements: (1) the nature of the substrate or phase on or in which the cells are to be cultivated; (2) the composition and concentration of the gaseous atmosphere in which the cells will be incubated; (3) the temperature of incubation; and (4) the physiochemical and physiological constitution of the culture medium (Freshney, R.I., In: Culture of Animal Cells, a Manual of Basic Technique, Alan R. Liss, Inc. N.Y. pp55-78 (1983); Lambert, K.J., et al., In: Animal Cell BioTechnology, Vol. 1, Spier, R.E., et al., Eds., Acad. Press N.Y. pp86-122 (1985) which references are hereby incorporated by reference). Thus, important considerations include whether it is desirable to cultivate the cells in a suspension of culture medium or to permit them to grow in contact with a solid surface. Suitable solid surfaces include glass, polysty-

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rene culture dishes, or the surfaces of suspendible particulates. Alternatively, the cells may be grown in permeable membraneous spheres or in hollow fibers. Although any cultivation technique capable of maintaining the cellular viability or proliferative growth capacity of PBL cells may be employed, it is preferable to cultivate such cells in a suspension in T-flasks or roller bottles (Mazumder, A., et al., Cancer 53:896-905 (1984)), although other methods, such as growth in air-lift or deep tank fermenters or hollow fiber, ultra-filtrative devices, may also be suitable. The volume of culture medium may be as little as 50 ul or as much as 100 liters.

Although the cells may be incubated at any temperature in which cellular viability may be sustained, it is preferable to maintain cells at temperatures between 30 and 45°C and it is most preferable to cultivate the cells at an incubation temperature of 37°C. Cultured cells will, however, tolerate considerable drops in temperatures and can survive several days at 4°C and can be frozen and cooled to -196°C in the presence of suitable cryopreservatives. In regulating the temperature of cultured cells, consistency of temperature maintenance is important; the temperature should be maintained within $\pm 2^\circ\text{C}$ of the desired temperature.

The gaseous atmosphere in which the cells are incubated must contain oxygen, and carbon dioxide. Although numerous gaseous compositions and concentrations are possible, it is preferable to employ a gaseous atmosphere containing air which has been supplemented to contain a final concentration of 5% carbon

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dioxide. In addition, the atmosphere should be of a relative humidity greater than 80% and preferably greater than 90%.

The basic constituents of a general tissue culture medium are disclosed by Waymouth, C. (In: Methods for Preparation of Media, Supplements and Substrata for Serum-Free Animal Cell Culture, Alan R. Liss, NY, pp23-68 (1984)) which is hereby incorporated by reference. The basic components of a culture medium include the ions of inorganic salts (such as, for example, Na^+ , K^+ , Ca^{2+} , Mg^{2+} , Cl^- , HPO_4^{2-} and HCO_3^-). In addition, a culture medium may contain metal ions (such as, for example, Fe^{2+} , Zn^{2+} , Cu^{2+} , Mn^{2+} , Co^{2+} and SeO_3^{2-}). Additionally, culture media may contain trace amounts of elements such as Sn, V, Cr, Al, As, and Mo.

The culture medium of the invention may also contain amino acids, such as those which naturally occur in animal protein. Vitamins, such as (for example): B_{12} , C, A, E, and biotin, as well as lipids and fat soluble components are also generally included in cultured medium. Lipids and fat soluble components which have been used in culture media include linoleic acid, cholesterol, lecithin, sphingomyelin, phospholipids, and oleic acid, among others.

The culture media of the invention also contain carbohydrates which serve as sources of energy. Although it is preferable to employ glucose and/or glutamine as the principle energy source in the media of the present invention, other sugars (such as fructose, galactose, mannose, maltose or lactose) may also be employed. The concentrations of sugars in such media generally exceeds 1000 mg/L and may exceed 5000 mg/L.

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Although the cells of the invention may be capable of synthesizing nucleotides de novo in in vitro, the addition of purine or pyrimidine bases or nucleotides may increase growth efficiency. The bases that may be added to the serum-free media of the invention include adenine and hypoxanthine. Nucleotides include thymidine, deoxyadenosine, deoxycytidine, and deoxyguanosine.

PBL cells are sensitive to changes in the pH of the culture medium. Thus, it is necessary to provide pH buffers to cultured media. Among the buffers which may be used are HEPES, bicarbonate buffers, phosphate buffers, tris buffers, other similar buffers. It is desirable that such buffers be capable of maintaining a pH of approximately 6.5-8.0, and preferably a pH between 6.8 - 7.4.

It is also desirable to supplement the culture media with growth factors, mitogens, or hormones. Among those, growth factors and hormones which may be added include insulin, cortisol or dexamethasone, Interleukin 1, interferons, transferrin, etc. In addition, it is often desirable to include human albumin to the culture medium.

Since PBL cells grow more slowly than potential contaminants, such as bacteria, it may be desirable to supplement the culture medium with antibiotics such as penicillin, streptomycin, gentamycin, etc.

Although it is possible, by routine experimentation, to develop a cell culture medium containing suitable amounts of the above described components, it is alternatively possible to employ any of a wide number of commercially prepared cultural media in which

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the specific combinations and concentrations of components have already been determined. The compositions of such culture media are disclosed by Freshney, R.I. In: Culture of Animal Cells a Manual of Basic Technique, Alan R. Liss, NY, pp67-78 (1983)).

A "basal medium," as used herein is a medium which contains basic components of the culture medium (such as those described above). Such basal medium (and also the LAK cell-generating medium disclosed herein) is said to be "substantially free of serum" if the concentration of serum is less than 0.1%. Although numerous possible culture media may be formulated which are capable of serving as the basal medium of the present invention, it is especially preferable to employ a basal medium which consists of Dulbecco's Modified Eagle's Medium: Ham's F12 medium in a 1:1, by volume, ratio (Barnes, D. et al., Anal. Biochem. 102:1255 (1980)). Such medium is hereinafter referred to as Darfler's Basal Medium. The compositions of these media are disclosed in Table 1.

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TABLE I

Component	Dulbecco's modification mg/l	Ham's F12 mg/l	Darfler's Basal Medium mg/l
Inorganic salts			
CaCl ₂ (anhyd.)	200.00	---	100.00
CaCl ₂ ·2H ₂ O	---	44.00	22.00
Fe(NO ₃) ₃ ·9H ₂ O	0.10	---	.05
KCl	400.00	223.60	311.80
MgCl ₂ ·6H ₂ O	---	122.00	61.00
MgSO ₄ ·7H ₂ O	200.00	---	100.00
NaCl	6,400.00	7,599.00	6,999.50
NaHCO ₃	3,700.00	1,176.00	2,438.00
NaH ₂ PO ₄ ·H ₂ O	125.00	---	62.50
NaHPO ₄ ·7H ₂ O	---	268.00	134.00
CuSO ₄ ·5H ₂ O	---	0.00249	0.00125
FeSO ₄ ·7H ₂ O	---	0.834	0.417
ZnSO ₄ ·7H ₂ O	---	0.863	0.432
Other Components			
D-glucose	4,500.00	1,802.00	3,150.00
Lipoic acid	---	0.21	.11
Phenol red	15.00	12.0	13.50
Sodium pyruvate	110.00	110.00	110.00
Hypoxanthine	---	4.10	2.05
Linoleic acid	---	0.084	0.042
Putrescine 2HCl	---	0.161	0.081
Thymidine	---	0.73	0.37
Amino Acids			
L-alanine	---	8.90	4.45
L-arginine.HCl	84.00	211.00	147.50
L-asparagine.H ₂ O	---	15.01	7.50
L-aspartic acid	---	13.30	6.65
L-cystine	48.00	---	24.00
L-cysteine.HCl.H ₂ O	---	35.12	17.56
L-glutamic acid	---	14.70	7.35
L-glutamine	584.00	146.00	365.00
Glycine	30.00	7.50	18.75
L-histidine HCl.H ₂ O	42.00	20.96	31.48
L-isoleucine	105.00	3.94	54.47
L-leucine	105.00	13.10	59.05
L-lysine HCl	146.00	36.50	91.25
L-methionine	30.00	4.48	17.24
L-phenylalanine	66.00	4.96	35.48
L-proline	---	34.50	17.25
L-serine	42.00	10.50	26.25
L-threonine	95.00	11.90	53.45

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TABLE 1 (cont.d)

Component	Dulbecco's modification mg/l	Ham's F12 mg/l	Darfler's Basal Medium mg/l
L-tryptophane	16.00	2.04	9.02
L-tyrosine	72.00	5.40	38.70
L-valine	94.00	11.70	52.85
Vitamins			
Biotin	---	0.0073	.0037
D-Ca pantothenate	4.00	0.4800	2.24
Choline chloride	4.00	13.9600	8.98
Folic acid	4.00	1.300	2.65
i-inositol	7.20	18.000	12.60
Nicotinamide	4.00	0.04	2.02
Pyridoxal HCl	4.00	0.062	2.03
Riboflavin	0.40	0.0380	.22
Thiamine HCl	4.00	0.3400	2.17
Vitamin B ₁₂	---	1.3600	.68
Pyridoxine HCl	---	0.0620	.032

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II. The Generation of Lymphokine-Activated Killer cells.

Cellular immune reactions result from complex interactions between T cells, B cells and macrophages. T cells are "white blood cells" which are formed in the thymus gland. They produce and respond to a variety of different stimuli (such as lymphokines and antigens) to control and modulate an animal's immune response. B cells, which are formed in bone marrow tissue, are the "white blood cells" which produce antibodies in response to infection. Macrophages are the white blood cells which are primarily responsible for removing foreign cells (i.e. bacteria, etc.) from an animal's tissues. The complex interactions between these cells are mediated through direct cellular contact and through soluble compounds known as "lymphokines." Lymphokines are, in general, protein or glycoproteins having a molecular weight between 10-100 kd. Examples of lymphokines are disclosed by Stephenson (In: Animal Cell Biotechnology, Vol. 2, Spier, R.E., et al., eds., Acad. Press, pp 41-45 (1985)). Although any compound capable of directing the activation of PBL cells into LAK cells may be employed according to the present invention, it is preferable to employ a lymphokine, and it is especially preferable to employ the lymphokine, interleukin-2 (IL-2). Interleukin-2 is a 15 kd glycoprotein (Gillis, S., et al., J. Exper. Med. 152:1709 (1980)) which is secreted by activated T lymphocytes (Bonnard, et al., J. Immunol. 123:2704 (1979)). This molecule is believed to interact with a membrane receptor present on activated lymphocytes thereby inducing a cascade

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phenomena which lead to the proliferation of the activated cells (Robb, J.R., Immunol. Today 7:203 (1984)).

Lymphokines may be purified from natural tissue sources (Spiess, P.J., et al., J. Immunol. Meth. 42:213-222 (1981)), or may be obtained through recombinant DNA techniques (Rosenberg, S.A., et al., Science 223:1412-1414 (1984)) Nishimura, T. et al., Canc. Immunol. Immunother. 21:12 (1986)).

As disclosed above, lymphokines are capable of converting a subset of peripheral blood lymphocytes into LAK cells. Lymphocytes characteristically have a round nucleus with well-condensed chromatin. Methods for preparing and purifying lymphocytes are disclosed by Freshney, R.I. (In: Culture of Animals Cells, A Manual of Basic Technique, Alan R. Liss, Inc., N.Y., p238 (1983) and by Mazumder, A., et al., Cancer, 53:896-905 (1984)).

As indicated, a lymphokine activated killer cell is a peripheral blood lymphocyte cell which has been activated by contact with a lymphokine. Although the precise mechanism through which LAK cells are produced is not fully understood, such an understanding is not necessary in order to practice the invention since it is known to be possible to spontaneously generate LAK cells by incubating PBL cells, under appropriate in vitro tissue culture conditions in the presence of a lymphokine. The amount of lymphokine exogenously added to the culture medium is typically determined by diluting a stock solution of the lymphokine and determining the lowest amount of lymphokine capable of inducing LAK cell formation (Suzuki, R., et al., J. Immunol. 130:981 (1983), Handa, K. et al., J. Immunol.

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130:988 (1983)). As little as three units/ml and as much as 1,000 units/ml of interleukin-2 can be used to produce LAK cells from PBL cells. A unit of interleukin-2 is defined as the reciprocal of the dilution that induces 50% of maximal [³H] TdR incorporation of NK-7 cells. (Itoh, K., et al., J. Immunol. 134:1345-1349 (1985)).

A compound is said to be "exogenously added" when its concentration in a cell or medium has been increased through the addition of the compound by one practicing the invention. This addition may either be direct, as by adding the compound itself, or may be indirect, as by adding a precursor of the compound. A compound which is normally and naturally present either within a cell or in a culture medium is not said to be exogenously added to the medium unless its concentration has been increased in a manner consistent with the above definition.

The incubation of PBL cells should be for at least one day (Itoh, K., et al., (1985)), however, the percentage of PBL cells which become activated to LAK cells increases with increasing time. It is preferable to culture PBL cells when they are an initial concentration of between 10^6 - 8×10^6 cells/ml. Generally it is not preferable to subculture the cells (i.e. to divide the culture's concentration of cells by adding additional, but sterile, media). It is desirable to maintain LAK-inducing conditions for 3-10 days. Once LAK cells have been produced, the cells may be cultivated in vitro for periods longer than two months (Rayner, A.A., et al., Cancer, 55:1327-1333 (1985)). Under these conditions, it is preferable to

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subculture LAK cells so as to maintain a cell concentration of between 10^4 - 10^8 cells/ml. Since LAK cells, thus expanded, may potentially increase more than 10^{20} - fold, LAK cell therapy may be employed even in individuals (such as cancer patients) from whom only small numbers of PBL cells may be obtained. Although interleukin-2 is capable of generating LAK cells without the addition of any other lymphokine, the addition of additional lymphokines (such as gamma interferon) has been found to augment IL-2-induced LAK generation (Shiiba, K., et al., Cancer Immunol. Immunother. 21:119-128 (1986)).

LAK cells are capable of attacking a diverse group of tumors (Grimm, E.A., et al., J. Exper. Med., 155:1823-1841 (1982)). Among the types of tumors thought to be treatable with LAK cell therapy are soft tissue sarcomas, melanomas, osteosarcoma, adenocarcinoma (such as colon, ovary, and pancreatic carcinomas) lymphomas, esophageal carcinomas and adrenal carcinomas (Rayner, A.A., et al., Cancer 55:1327-1333 (1985)).

An important aspect of the present invention is the discovery that non-proteinaceous compounds, capable of activating the endogenous enzyme kinase C, are capable of promoting the generation of LAK cells from PBL cells which have been cultivated in a serum-free medium in the presence of a lymphokine. A "non-proteinaceous" compound is one which is not composed of amino acids linked together through a peptide bond. Any non-proteinaceous compound capable of activating kinase C may be employed to accomplish this goal. Examples of such activator compounds are acetylacetyl-glycerols, and diacylglycerols (Ebeling, J.G. Proc.

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Natl. Acad. Sci. USA, 82:815-819 (1985); Mufson, R.A., Carcinogenesis, 6:1693-1698 (1985)). The synthesis of diacylglycerols which activate kinase C is disclosed by Ebeling, J.G., et al., (1985).

Especially preferred are the diacylglycerols. Although it is possible, in accord of the present invention to employ either a racemic mixture of diacylglycerols or one containing unequal amounts of the diacylglycerol's stereoisomers, it is preferable to employ the sn-1,2-diacylglycerols. Especially preferred sn-1,2-diacylglycerols are those with short saturated acyl side chains (containing between 2 and 12, and preferably between 4 and 10 carbon atoms). According to the present invention, the diacylglycerol, sn-1,2-dioctanoylglycerol (diC₈), which has an 8 carbon long acyl side chain, was the most preferred reagent for promoting LAK cell generation and proliferation. This compound is capable of mediating such an effect when present at a concentration greater than 1 mg/L, however it is more preferable to employ this reagent at a concentration of between 0.5-20 mg/L and most preferable to employ the reagent at a concentration of 5-20 mg/L. In general, this compound is only slightly soluble in water (3-5 mg/L). The compound's solubility can, however, be increased to 10-20 mg/L through the use of solubilizing agents such as Tween (at a concentration of between 1-20 mg/L) or Albumin (at a concentration of between 1000-10000 mg/L.)

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Various methods can be used in order to determine whether a particular compound is an activator of Kinase C. Platelets, for example, can be prelabeled with ^{32}P and then incubated with the compound for 30 seconds. The platelet cells can then be lysed and the proteins liberated from them can be analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (using, for example, an 11% polyacrylamide gel) and autoradiography. The presence of a radiolabeled 40,000 - dalton protein indicates that the potential activator compound is, indeed, capable of activating kinase C. This method is described by Lapetina, et al. (J. Biol. Chem., 260:135-136 (1985)). Alternatively, the ability of a compound to activate kinase C can be assayed using cell-free kinase C - containing cellular extracts or purified kinase C (in the presence of buffer, MgCl_2 , CaCl_2 , H_1 Histone (substrate), [γ - ^{32}P] ATP and phosphatidyl serine. This method is described by Kraft, A.S. et al. (J. Biol. Chem., 257:13193 (1982)). Any compound capable of activating kinase C (as determined, for example, by either of the above-described methods) may be employed as the kinase C activating compound of the present invention.

According to the present invention, the activation of kinase C by any means will permit the development of PBL cells into LAK cells. Thus, as an alternative to the direct activation of kinase C it may be possible to accomplish the goal of the present invention by including compounds which indirectly activate the kinase C enzyme. Such substances include, for example, agents which hydrolyze phosphatidyl inositol

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(thus producing a diacylglycerol), or by including compounds structurally similar to the activator compound, which are capable of mediating kinase C activation. Further, it may be possible to employ as activating agents precursor compounds (such as triglycerides) which are diacylglycerol precursors (In: Fundamentals of Clinical Chemistry N.W. Tietz Ed., W.B. Saunders Co., pp416-417 (1970)). An example of such a triglyceride is the compound tricaprylin.

Additionally, any analogs of diacylglycerols (such as 1,2-dicaprin, 1,2-dicaproin and 1,2-dicaprylin) or acetylacylglycerols (such as 1-oleoyl-2-acetyl-glycerol) which are capable of activating kinase C may be employed as the activating agent of the present invention.

Having now generally described this invention, the same will be better understood by reference to certain specific examples which are included herein for purposes of illustration only and are not intended to be limiting of the invention, unless specified.

EXAMPLE I

The Preferred Medium for the Generation of LAK Cells

The Darfler Basal Medium described above and in Table 1 was prepared and supplemented with the following: 50 U/ml penicillin, 50 ug/ml streptomycin, 10 ug/ml gentamycin, 3 mg/L ethanolamine, 2 mg/L human insulin, 5 mg/L human transferrin, 2500 mg/L human albumin (fraction V or fatty acid-free fraction V), 1

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mg/L indomethacin (fatty acid-free), 0.003 mg/L sodium selenite, 2600 mg/L HEPES buffer, (pH 7.0), 2500 mg/L sodium bicarbonate, 3500 mg/L L-glutamine, 55 mg/L pyruvate (sodium salt), 1.5 mg/L cholesterol, 3 mg/L dilinoleoylphosphatidyl choline, 1 mg/L linoleic acid, 1 mg/L palmitic acid, 5mg/L Tween 80 (polyoxyethlenesorbitan, monooleate). Water was added to the medium to bring the osmolality to 280-300 mOsm. Immediately prior to use, 600 U/ml of recombinant interleukin-2 was added to the medium. As used below, when Darfler's Basal Medium is supplemented with all of the above described compounds except for the non-proteinaceous activator of kinase C it is referred to as "Darfler's Supplemented Medium." When the activator compound is added to Darfler's Supplemented Medium the medium is then referred to as "Darfler's Complete Medium" or, alternatively, as Darfler's Supplemented Medium with a disclosed amount of activator compound.

EXAMPLE II

Cultivation of Peripheral Blood Lymphocytes in a Serum-Free Medium

Human peripheral blood lymphocytes were purified by the method of Mazumder, A. et al. (Cancer, 53:896-905 (1984)). The cells were cultured at a concentration of 2×10^6 cells/ml in 2 ml. of Darfler's Complete Medium in 25 cm² T flasks in a 100% humidity, 5% CO₂ environment at 37°C. After 7 days, the cells were counted by hemacytometer and assayed for cytotoxicity against Daudi cells using a Cr release assay according to the method of Mazumder, et al. (1984). By this process a culture of PBL cells was established.

EXAMPLE IIIDiacylglycerol-Containing Serum-Free Medium
Allows the Expansion of LAK Cells in Serum-Free
Medium in a Seven Day Incubation

PBL cells obtained as described in Example II were grown according to the method of Mazumder, A., *et al.* (Cancer 53:896-905 (1984)) in RPMI-1640 medium supplemented with 2% human serum or were incubated in either Darfler's Supplemented Medium (described in Example I) or Darfler's Supplemented Medium containing 20 mg/L diC_8 and incubated for seven days at 37°C in an atmosphere containing 95% air and 5% CO_2 . After this incubation, the cytotoxicity of the cultured cells was determined. This experiment revealed that the incubation of PBL cells in serum-free medium which contained a diacylglycerol was able to produce LAK cells at 97% of the efficiency at which such cells were generated after incubation in serum-containing medium. In contrast, serum-free medium which lacked diacylglycerols produced LAK cells at only 1/3 the efficiency of serum-containing medium. These results therefore indicate that a serum-free medium containing diacylglycerols is capable of replacing serum-containing medium for the generation of LAK cells, and that such a serum-free medium is capable of generating LAK cells at an efficiency similar to that of a serum-containing medium.

EXAMPLE IVAmounts of Interleukin-2 Required for
LAK Cell Production

PBL cells were prepared as described in Example II and divided into four aliquots which were subsequently incubated in either 98% RPMI-1640 medium supplemented with 2% serum (Mazumder, A., et al. (1984)) or in Darfler's Complete Medium containing the diacylglycerol, sn-1,2-dioctanoylglycerol at 20 mg/L. The Darfler Complete Medium contained either 100, 300, or 1000 U/ml of interleukin-2. After a seven day incubation at 37°C in an atmosphere of 95% air, 5% carbon dioxide the relative efficiency of LAK cells proliferation was determined. Serum-free medium containing 1000 units/ml interleukin-2 was found to produce LAK cells which lysed target cells at 52% the efficiency with which such cells were produced by incubation in serum-containing medium which contained 1000 U/ml interleukin-2. Serum-free medium containing 300 or 100 U/ml interleukin-2 were found, however, to produce LAK cells at an efficiency which was 106% and 85%, respectively, of that observed in the serum-containing medium control. This experiment shows that the use of serum-free medium containing diacylglycerols permits LAK cell formation to occur in the presence of lower amounts of interleukin-2 than are required for LAK cell formation in serum-containing media. Such a result is significant in that the availability and cost of interleukin-2 poses a significant obstacle to the use of LAK-cell therapeutic regimens.

EXAMPLE VInhibitors of Diacylglycerol Breakdown
Enhance LAK Cell Production

PBL cells were prepared as described in Example II and divided into two aliquots which were subsequently incubated in Darfler's Complete Medium (containing sn-1,2-diocanoylglycerol at 5 mg/L, 1000 U/mL interleukin-2, an additional 10 mg/L Tween 80, and an additional 1.5 mg/L cholesterol) in the presence or absence of 1-monooleoyl-rac-glycerol at 10 mg/L. After seven days incubation at 37°C in an atmosphere of 95% air, 5% carbon dioxide the relative efficiency of LAK cells to lyse target cells was determined. In the absence of 1-monooleoyl-rac-glycerol the percent lysis was 35 at an effector:target of 8:1, 21 at 4:1 and 13 at 2:1. In contrast, in the presence of 1-monooleoyl-rac-glycerol the percent lysis was 50, 33 and 25, respectively. Similar results were obtained in two other experiments, with levels of 1-monooleoyl-rac-glycerol being optimal at 5-10 mg/L. 5 mg/L was found to be the most preferred concentration of monooleoyl-rac-glycerol. Diacylglycerols and their derivatives breakdown due to the action of enzymes such as diacylglycerol kinase and diacylglycerol lipase. Agents (i.e., chemical compounds) which inhibit this breakdown result in higher intercellular levels of diacylglycerols and their derivatives. Since 1-monooleoyl-rac-glycerol inhibits diacylglycerol kinase and diacylglycerol lipase (W.R. Bishop et al., J. Biol. Chem. 261:12513-12519 (1986) and W.R. Bishop et al., J. Biol. Chem., 261:6993-7000 (1986)), these data

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suggest that the inhibition of these enzymes in cells acts to elevate intracellular levels of diacylglycerol. As a consequence, greater numbers of LAK cells, greater killer activity, or both is generated by the inclusion of 1-monooleoyl-rac-glycerol. Other inhibitors of diacylglycerol breakdown which may have similar activity include, but are not limited to, dioctanoylethylene glycol, dioctanoyl glyceramide, dioctanoyl butanetriol and other analogs of diacylglycerol and monoacylglycerol.

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure have come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features herein before set forth and as follows in the scope of the appended claims.

WHAT IS CLAIMED IS:

1. A method for achieving the proliferative growth of a peripheral blood lymphocyte cell or a lymphokine activated killer cell, which method comprises:

- (a) contacting said cell with an amount of a culture medium sufficient to permit the in vitro cultivation of said cell, said medium being substantially free of serum and containing a non-proteinaceous compound capable of activating the enzyme kinase C; said compound being present in said medium at a concentration sufficient to direct said activation, and
- (b) maintaining said cell in said culture medium under conditions sufficient to produce said proliferative growth.

2. A method for producing a lymphokine activated killer cell which comprises:

- (a) contacting a peripheral blood lymphocyte cell with an amount of a culture medium sufficient to permit the in vitro cultivation of said peripheral blood lymphocyte cell, said medium being substantially free of serum and containing (i) a lymphokine and (ii) a non-proteinaceous compound capable of activating the enzyme kinase C; said compound being present in said medium at a concentration sufficient to direct said activation, and

WHAT IS CLAIMED IS:

1. A method for achieving the proliferative growth of a peripheral blood lymphocyte cell or a lymphokine activated killer cell, which method comprises:

- (a) contacting said cell with an amount of a culture medium sufficient to permit the in vitro cultivation of said cell, said medium being substantially free of serum and containing a non-proteinaceous compound capable of activating the enzyme kinase C; said compound being present in said medium at a concentration sufficient to direct said activation, and
- (b) maintaining said cell in said culture medium under conditions sufficient to produce said proliferative growth.

2. A method for producing a lymphokine activated killer cell which comprises:

- (a) contacting a peripheral blood lymphocyte cell with an amount of a culture medium sufficient to permit the in vitro cultivation of said peripheral blood lymphocyte cell, said medium being substantially free of serum and containing (i) a lymphokine and (ii) a non-proteinaceous compound capable of activating the enzyme kinase C; said compound being present in said medium at a concentration sufficient to direct said activation, and

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(b) maintaining said cell in said culture medium under conditions sufficient to permit the production of said lymphokine activated killer cell.

3. The method of any one of claims 1 and 2 wherein said compound capable of activating the enzyme kinase C is selected from the group consisting of a diacylglycerol, a functional analogue of a diacylglycerol, and a precursor of a diacylglycerol.

4. The method of any one of claims 1 and 2 wherein said compound capable of activating the enzyme kinase C is selected from the group consisting of an acetylacylglycerol, a functional analogue of an acetylacylglycerol, and a precursor of a acetylacylglycerol.

5. The method of claim 3 wherein said compound capable of activating the enzyme kinase C is a diacylglycerol.

6. The method of claim 5 wherein said diacylglycerol contains a side chain of between 6-10 carbons in length.

7. The method of claim 6 wherein said diacylglycerol is selected from the group consisting of sn-1,2-dioctanoylglycerol and sn-1,2-dihexanoylglycerol and sn-1,2-didecanoylglycerol.

8. The method of claim 7 wherein said diacylglycerol is sn-1,2-dioctanoylglycerol.

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9. The method of any one of claims 1 and 2 wherein said culture medium is Darfler's Complete Medium.

10. The method of any one of claims 1 and 2 wherein said compound capable of activating the enzyme kinase C is present at a concentration of from 1-20 mg/L.

11. The method of claim 10 wherein said compound capable of activating the enzyme kinase C is present at a concentration of from 5-20 mg/L.

12. A method for suppressing the growth or development of a cancer cell which comprises:

- a) contacting a peripheral blood lymphocyte cell with an amount of a culture medium sufficient to permit the in vitro cultivation of said cell, said medium being substantially free of serum and containing (i) a lymphokine and (ii) a non-proteinaceous compound capable of activating the enzyme, kinase C; said compound being present at a concentration sufficient for said activation,
- b) maintaining said cell in said culture medium under conditions sufficient to permit the development of a lymphokine activated killer cell, and
- c) providing said lymphokine activated killer cell to said cancer cell in a manner sufficient to suppress the growth or development of said cancer cell.

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13. The method of claim 12 wherein said compound capable of activating the enzyme kinase C is selected from the group consisting of a diacylglycerol, a functional analogue of a diacylglycerol, and a precursor of a diacylglycerol.

14. The method of claim 12 wherein said compound capable of activating the enzyme kinase C is an acetylacetyl-glycerol, a functional analogue of an acetylacetyl-glycerol, or a precursor of an acetylacetyl-glycerol.

15. The method of claim 13 wherein said compound capable of activating the enzyme kinase C is a diacylglycerol.

16. The method of claim 15 wherein said diacylglycerol contains a side chain of between 6-10 carbons in length.

17. The method of claim 16 wherein diacylglycerol is selected from the group consisting of sn-1,2-dioctanoylglycerol, sn-1,2-dihexanoylglycerol and sn-1,2-didecanoylglycerol.

18. The method of claim 16 wherein said diacylglycerol is sn-1,2-dioctanoylglycerol.

19. A composition of matter which comprises (a) an in vitro cultivated peripheral blood lymphocyte cell or a lymphokine activated killer cell, (b) an exogenously added non-proteinaceous compound capable of activating the enzyme kinase C, and (c) a lympho-

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kine; wherein said composition is substantially free of serum.

20. The composition of matter of claim 19 wherein said exogenously added compound (b) is selected from the group consisting of a diacylglycerol, a functional analogue of a diacylglycerol, and a precursor of a diacylglycerol.

21. The composition of matter of claim 19 wherein said exogenously added compound (b) is selected from the group consisting of an acetylacylglycerol, a functional derivative of an acetylacylglycerol, and a precursor of an acetylacylglycerol.

22. The composition of matter of claim 20, wherein said exogenously added compound is a diacylglycerol.

23. The composition of matter of claim 22 wherein said diacylglycerol contains a side chain of between 6-10 carbons in length.

24. The composition of matter of claim 23, wherein said diacylglycerol is selected from the group consisting of: sn-1,2-dioctanoylglycerol and sn-1,2-dihexanoylglycerol and sn-1,2-didecanoylglycerol.

25. The composition of matter of claim 24 wherein said diacylglycerol is sn-1,2-dioctanoylglycerol.

26. A culture medium which contains an exogenously added non-proteinaceous compound capable of activating the enzyme kinase C; wherein said culture medium is substantially free of serum.

27. The culture medium of claim 26 wherein said compound capable of activating the enzyme kinase C is selected from the group consisting of a diacylglycerol, a functional analogue of a diacylglycerol, and a precursor of a diacylglycerol.

28. The culture medium of claim 26 wherein said compound capable of activating the enzyme kinase C is selected from the group consisting of an acetylacylglycerol, a functional analogue of an acetylacylglycerol, or a precursor of an acetylactlglycerol.

29. The culture medium of claim 27 wherein said compound capable of activating the enzyme kinase C is a diacylglycerol.

30. The culture medium of claim 29 wherein wherein said diacylglycerol contains a side chain of between 6-10 carbons in length.

31. The culture medium of claim 30 wherein said compound is selected from the group consisting of sn-1,2-dioctanoylglycerol and sn-1,2-dihexanoylglycerol and sn-1,2-didecanoylglycerol.

32. The culture medium of claim 31 wherein said diacylglycerol is sn-1,2-dioctanoylglycerol.

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33. The method of any of one of claims 1, 2, and 12 wherein said culture medium additionally contains an agent capable of inhibiting breakdown of said non-proteinaceous compound.

34. The method of claim 33 wherein said non-proteinaceous compound is selected from the group consisting of a diacylglycerol, a functional analogue of a diacylglycerol, and a precursor of a diacylglycerol.

35. The method claim 33 wherein said non-proteinaceous compound is selected from the group consisting of an acetylacylglycerol, a functional analogue of an acetylacylglycerol, or a precursor of an acetylacylglycerol.

36. The method of claim 33 wherein said agent is an inhibitor of an enzyme selected from the group consisting of diacylglycerol kinase and diacylglycerol lipase.

37. The method of claim 33 wherein said agent is selected from the group consisting of 1-monooleoyl-rac-glycerol, dioctanoylethylene glycol, dioctanoyl glyceramide, and dioctanoyl butanetriol.

38. The method of claim 37 wherein said agent is 1-monooleoyl-rac-glycerol.

INTERNATIONAL SEARCH REPORT

International Application No. **PCT/US87/02721**

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ¹ According to International Patent Classification (IPC) or to both National Classification and IPC IPC(4): C12N 5/02 U.S.Cl.: 435/240.25, 240.31, 424/93						
II. FIELDS SEARCHED <div style="text-align: center; font-size: small;">Minimum Documentation Searched ⁴</div> <table style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 30%; text-align: left; font-size: small;">Classification System</th> <th style="text-align: left; font-size: small;">Classification Symbols</th> </tr> <tr> <td style="vertical-align: top;">U.S.</td> <td style="vertical-align: top;">435/240, 240.1, 240.2, 240.25, 240.31 424/93</td> </tr> </table> <div style="text-align: center; font-size: small; margin-top: 10px;">Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁴</div> <p>Chemical Abstracts (CAS) 1967-1987; Biological Abstracts DATA BASE (BIOSIS) 1967-1987, APS 1975-1987 (See Attachment for Search Terms).</p>			Classification System	Classification Symbols	U.S.	435/240, 240.1, 240.2, 240.25, 240.31 424/93
Classification System	Classification Symbols					
U.S.	435/240, 240.1, 240.2, 240.25, 240.31 424/93					
III. DOCUMENTS CONSIDERED TO BE RELEVANT ¹⁴						
Category ⁸	Citation of Document, ¹⁶ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No. ¹⁸				
Y	Journal of Immunological Methods, North-Holland Amsterdam, Netherlands (Amsterdam, Netherlands), Volume 86, Issued 1986, C.J. Froelich et al, "Induction of lymphokine activated killer cells in serum-free medium". Entire document p205-211.	1-38				
Y	Journal of Biological Chemistry, American Society for Biochemistry and Molecular Biology (Baltimore, Maryland, USA) Volume 260(3), Issued 10 February 1985, K. Kaibuchi et al, "Protein Kinase C and Calcium ion in mitogenic response of macrophage-depleted human peripheral lymphocytes" p. 1366-1369 especially pages 1366 and 1368-1369.	1-11				
Y	Animal Cell Biotechnology, Academic Press (New York, New York, USA), Volume 1, Issued 1985, Lambert et al, "Cell growth media" pages 85-122. Entire document.	9				
<div style="font-size: x-small;"> ⁸ Special categories of cited documents: ¹⁵ "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such docu- ments, such combination being obvious to a person skilled in the art. "Δ" document member of the same patent family </div>						
IV. CERTIFICATION						
Date of the Actual Completion of the International Search ¹ 5 JANUARY 1988 International Searching Authority ¹ ISA/US	Date of Mailing of this International Search Report ¹ <div style="font-size: large; font-weight: bold;">24 FEB 1988</div> Signature of Authorized Officer ¹⁹ <div style="text-align: center;"> Gail F. Knox </div>					

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No. 1*
Y	Cancer Immunology Immunotherapy, Springer (New York, New York, USA), Volume 21, Issued 1986, Shiiba et al, "Interleukin 2-activated killer cells: generation in collaboration with interferon and its suppression in cancer patients p. 119-128. Entire document.	12-18
Y	Journal of Biological Chemistry, (Baltimore, Maryland, USA), Volume 261(15), Issued 25 May 1986, Bishop et al, "Attenuation of Sn-1,2-Diacylglycerol second messengers by Diacylglycerol kinase" p. 6993-6999 especially pages 6993, 6999.	33-38
Y	Journal of Biological Chemistry, (Baltimore, Maryland, USA), Volume 260(3), Issued 10 February 1985, Lapetina et al, "Exogenous sn-1,2-Diacylglycerols containing saturated fatty acids function as bioregulators of protein kinase C in human platelets" p. 1358-1361 especially pages 1358, 1360-1361.	1-38
Y	Biochemical and Biophysical Research Communications, Academic Press (New York, New York, USA), Issued 30 August 1985, Guy et al, "Synergism between diacylglycerols and calcium ionophore in the induction of human B cell proliferation mimics the inositol lipid polyphosphate breakdown signals induced by crosslinking surface immunoglobulin" p. 484-491 especially pages 484-487 and 490-491.	1-38
Y	Nature, MacMillian (London, England) Volume 315, issued 16 May 1985, Farrar et al, "Interleukin-2 stimulates association of protein kinase C with plasma membrane" p. 233-235. Entire document.	1-38
A+P	Journal of Biological Chemistry, (Baltimore, Maryland, USA), Volume 262, Issued 5 April 1987, Koyasu et al "Signals for activation and proliferation of murine T lymphocyte clones" pages 4689-4695. Entire document.	1-11

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Attachment to Form PCT/ISA/210, Part II.

II. FIELDS SEARCH SEARCH TERMS:

lymphocyte, killer cell, lac cell, lymphokine activated
killer cell, culture, diacylglycerol, acetylacetylgy-
cerol, dihexonylglycerol, dioctanoylglycerol, dideca-
noylglycerol, activated killer cell, inhibition,
suppression, tumor, carcinoma, neoplastic, prolifera-
tion, inventor's name.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No. is
Y	Proceedings of the National Academy of Science, USA, National Academy of Sciences (Washington, DC, USA), Volume 82. Issued February 1985, Ebeling et al, "Diacylglycerols mimic phorbol diester induction of leukemic cell differentiation" p. 815-819 especially pages 815-816 and 819.	1-38
Y	Journal of Experimental Medicine, (New York, New York, USA), Volume 155, Issued June 1982, Grimm et al, "Lymphokine-activated killer cell phenomenon lysis of natural killer-resistant fresh solid tumor cells by interleukin 2-activated autologous human peripheral blood lymphocytes" p. 1823-1841. Entire document.	12-18
Y	Journal of Experimental Medicine, Rockefeller Union Press (New York, New York, USA), Volume 158, Issued October 1983, Grimm et al, "Lymphokine-activated Killer Cell phenomenon III. Evidence that IL-2 is sufficient for direct activation of peripheral blood lymphocytes into lymphokine-activated killer cells" p. 1356-1361. Entire document.	2-25, 33-38
Y	Cancer Research, Volume 44, Waverly Press, Inc. (Baltimore, Maryland, USA), Issued May 1984, Rosenstein et al, "Lymphokine-activated killer cells: Lysis of fresh syngeneic natural killer-resistant murine tumor cells by lymphocytes cultured in interleukin 2" p. 1946-1953. Entire document.	12-18
Y	Cancer Treatment Reports, Government Printing Office (Bethesda, Maryland, USA), Volume 68(1), Issued January 1984, Rosenberg, "Adoptive Immunotherapy of Cancer: Accomplishment and Prospects" p. 233-255. Entire document.	12-18
Y	Journal of Experimental Medicine, Rockefeller Union Pres. (New York, New York, USA), Volume 159, Issued February 1984, Mazumder et al, "Successful immunotherapy of natural killer resistant established pulmonary melanoma metastases by the intervenous adoptive transfer of syngeneic lymphocytes activated in vitro by Interleukin 2". Entire document. p. 495-507.	12-18

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Y	Analytical Biochemistry, Academic Press (New York, New York, USA), Volume 102, Issued 1980, Barnes et al, "Methods for growth of cultured cells in serum- free medium" p. 255-270. Entire docu- ment.	9
Y	Nature, Macmillian Magazines (London, England), Volume 313, Issued 24 January 1985, Truneh et al, "Early steps of lymphocyte activation bypassed by synergy between calcium ionophores and phorbol ester p. 318-320. Entire docu- ment.	1-11

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE 10

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers _____, because they relate to subject matter ¹² not required to be searched by this Authority, namely:
2. ☐ Claim numbers _____, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out ¹³, specifically:

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING 11

This International Searching Authority found multiple inventions in this international application as follows:

- I. Claims 19-25, 1-11 and 33-38 are drawn to a cell-containing composition and method of making the composition classified for example in class 435, subclass 240.25;

(See Attachment)

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.
☐ No protest accompanied the payment of additional search fees.

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Attachment to Form PCT/ISA/210, Part VI.

VI. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING

Claims 26-32 are drawn to a culture medium per se, classified for example, in class 435, subclass 240.31.

II. Claims 12-18 are drawn to a method of using killer cells, classified for example in class 424, subclass 93.